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REVIEW

The Diagnostic Significance of C3d Antigen in Kidney and Skin Histopathology – The Current State-Of-The-Art and Practical Examples

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Summary

The aim of this narrative review is to summarize recent knowledge about the diagnostic significance of immunobiological detection of C3d with a focus on renal and skin tissue biopsies. We completed the present narrative review with our own experiences with preparation and practical use of monoclonal C3d antibodies at a small national level.

Key words

C3d • Immunohistochemistry • Kidney transplant • Renal tubular damage • Bullous pemphigoid • Cutaneous lupus erythematosus

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Introduction

The complement system is composed of more than 30 soluble serum proteins that are present as zymogens and activated in a cascade. These proteins are classically cataloged as part of the innate immune system. However, there is a large body of information indicating that this system bridges the innate and acquired immune responses. The main proteins in the bridging function are the products derived from the cleavage of the third component of complement (C3) – C3b, iC3b, and C3d [1]. The C3 has a central role in the complement cascade and has important pro-inflammatory and immunoregulatory functions [2]. Because tissue-bound C3 fragments are associated with local inflammation, they have also been exploited as addressable binding ligands for targeted therapeutics and diagnostic agents in several tissues, including the kidneys, the heart, the brain, and the eyes [3]. C3d is a terminal degradation product of C3 that plays an important role in the modulation of the adaptive immune response through the interaction with complement receptor type 2 (CR2). The C3d complement fragment is formed both in classic and alternative complement activation processes [4]. C3d, as the final degradation product of C3, is more stable *in vivo* than C3c, because C3d remains attached to the tissue site (binds covalently to cell surfaces) after recovery of injury leaving a visible footprint. Therefore, C3d would be in some cases a more sensitive and robust immunostaining marker than other C3 fragments [5].

C3d is associated with the pathogenesis of numerous diseases with different etiologies, especially infectious viral, inflammatory, and autoimmune diseases. For this reason, antibodies anti-C3d may be relatively new and useful markers in the histopathological diagnosis of some acute or chronic tissue injuries. Most recent papers are focused on the role of C3d antigen in renal and skin pathologies. The aim of this narrative review is to summarize recent knowledge about the diagnostic significance of immunobiological detection of C3d with

a focus on renal and skin tissue biopsies. We completed the present narrative review with our own experiences with preparation and practical use of monoclonal C3d antibodies at a small national level.

The role of C3d antigen in kidney transplantation and kidney diseases

The specialty of kidney transplantation has made dramatic strides over the decades evolving from an experimental procedure to the standard of care in the treatment of patients with end-stage renal disease. Nowadays, transplantation remains the optimal mode of replacement therapy for the vast majority of patients with end-stage kidney disease. Significant progress has occurred over the decades in renal transplantation and is mostly driven by improvements in short-term graft and patient survival, but unfortunately, long-term graft survival after one year has only been a little improved [6].

Rejection has always been a major obstacle to transplantations. Accumulating evidence suggests that innate immunity interacts with the adaptive immune system to identify potentially harmful antigens and eliminate them from the host [7]. In renal transplant, the allograft is responsible for triggering many innate and adaptive immune mechanisms, either mediated by cells, such as macrophages and lymphocytes or by soluble components, such as antibodies and the complement system, which can ultimately lead to graft rejection [8]. Transplantation of tissues or cells from a donor who differs genetically from the graft recipient induces an immune response in the recipient against alloantigens of the donor graft. If not controlled, this response will destroy the graft [9].

The deposition of complement factors in renal tissues is a well-known phenomenon in pathologic conditions mediated by immune mechanisms. The exact significance of C3d in the diagnostics of tissue rejection is not clear, which is due to its involvement in several pathways within complement activation. According to present knowledge, the complement system can be activated either *via* the classical pathway, i.e. with the deposition of immunoglobulins and interaction with the complement components C1, C2, and C4, or by the alternative pathway. Activation *via* the classical pathway has been suggested to occur in allografted kidneys with the deposition of C3, terminal complement complexes, S-protein, and immunoglobulins [10]. On the other hand, C3d is less specific than C4d, because it is generated also by the alternative complement activation pathway, triggered both by antibodies and by tissue injury, especially in case of an incorrect function of the mechanisms protecting the tissues against the host's complement system. It has not been completely resolved whether the presence of C3d depositions is a marker of a separate acute rejection type, or of a particular form of C4d rejection. Both these fragments can also be formed as a result of lectin-induced complement system activation, occurring in the course of infections caused by various microorganisms, as well as during post-reperfusion damage of the graft [4].

The kidney is the first organ whose acute rejection was investigated and defined thoroughly, and the role of C4d in the diagnostics of that process was confirmed by numerous studies and guidelines. For example, the Banff classification for allograft pathology has become the most commonly used classification system for renal allograft pathology [11]. There are less numerous studies focused on the role of C3d complement system fragment (in comparison to C4d) in kidney graft rejection. From the theoretical point of view, C3d deposits should be a better marker of acute humoral rejection as they reach higher concentrations after classic complement activation because of amplification and involvement of an alternative activation pathway. However, the kidneys can produce small amounts of C3d under physiological conditions which may complicate the presentation of the pathology [4]. In the transplanted kidney, C3 synthesis occurs in proximal tubular epithelial cells as well as other cell types. Production by these cells increases during transplant rejection and is regulated by cytokines. Indeed, rejecting human renal allografts can contribute as much as 16 % of the circulating C3 pool. However, until now the functional relevance of such local synthesis has been unclear [7]. Local synthesis of complement protein C3 from renal epithelial cells is an important mediator for local tissue injury in renal disease. Renal C3d deposition may play a role during an immunohistochemical examination of damaged tissues as an index of complement activation by all complement pathways [12].

In normal kidneys, C3d is immunofluorescently stained in a linear pattern along the glomerular basement membrane, and segmentally along the Bowman's capsule, tubular basement membrane, and arterioles. Immunoelectron microscopy of isolated basement membranes showed that C3d is localized exclusively on the epithelial side of the glomerular basement membrane.

These findings indicate that C3d is normally present in the glomeruli. In inflamed kidney tissue, the C3d staining tended to show an increase in both intensity and distribution [13]. Such interactions between C3d and components of the glomerular basement membrane could play important roles in complement-related pathological processes of the glomerulus [14].

Allograft biopsy remains the gold standard for diagnosing of multiple kidney diseases and rejection (although it is associated with morbidity) [15]. Presently, immunohistochemical staining of complement is universally considered a useful diagnostic procedure in the assessment of renal biopsies, where C3d has been shown to be a marker of humoral rejection. On the basis of compelling evidence that complement cascade is an important contributor to antibody-mediated graft destruction, stratification of the risk of graft loss has essentially relied on assays evaluating the ability of donor-specific antibodies to trigger complement activation. Detection of C4d deposition/C4d staining in renal capillaries historically represented the gold standard technique to detect complement activation. However, the results of several studies that assessed the performance of this assay in predicting the outcome of antibody-mediated kidney rejection have been contradictory [16,17]. In contrast to C4d, complement factor C3 is a less-specific product of both the classic and alternative complement cascade involved in glomerular diseases and only a few investigators have focused on the meaning of capillary C3 deposition in rejecting grafts [18]. But according to Pelletier *et al.* [19], identification of *de novo* post-transplant C3d binding donor-specific antibodies identifies those recipients at the highest risk of bot diffuse peritubular capillary C4d deposition on kidney biopsy and subsequent 5-year graft loss. This information may prove useful for targeting which patients and which donor-specific antibodies to treat following identification as well as recipient counseling regarding renal graft survival prognosis.

Other studies indicate that renal C3d (and not C4d deposition) could serve as a prognostic factor for graft functioning and survival in kidney transplant recipients with acute rejection. According to Kuypers *et al.* [18], the deposition of complement factor C3d in peritubular capillaries indicates a variant type of acute rejection characterized by a worse clinical outcome. Similarly, Lv *et al.* [20] in their longitudinal cohort study demonstrated that C3d deposition in the peritubular capillaries is closely related to renal dysfunction and pathological changes. Both cited studies concluded that deposition of C3d in peritubular capillaries is an indicator of poor prognosis, which can be used in clinical practice.

Immunohistochemical detection of C3d in diseased renal tissue has diagnostic potential in other renal diseases – especially based on autoimmune substrate, too. For example, Ma *et al.* [21] studied renal biopsies of patients with anti-glomerular basement membrane disease, which is a rare but life-threatening autoimmune disease. The detected C3d clearly in a linear or granular pattern along the glomerular capillary wall of all examined patients (even in those C3c negative cases). But no correlation was found between the intensity of C3d deposition and the clinical characteristics of patients. In this cited study, the general finding of C3d deposition on the capillary wall reflects the activation of the complement system during the renal damage process of human anti-glomerular basement membrane disease. C3d positive staining is also observed along the capillary walls and mesangial areas of patients with Pauci-immune necrotizing crescentic glomerulonephritis [22]. In this study, C3d positive staining was significantly associated with renal function (a higher median serum creatinine) and the need for dialysis at the onset of disease. Authors proposed C3d as a novel prognostic factor of end-stage renal disease and renal vasculitis. C3d is deposited also in the intima and the media of arterioles, presenting as the medial thickening and sclerosis of varying severities, in cases of IgA nephropathy. For this reason, Zhang *et al.* [23] described C3d as a useful marker for arteriolosclerosis in IgA nephropathy. Another possibility for the clinical use of antibodies anti-C3d in histopathological practice is the diagnosis of C3 glomerulopathy. C3-dominance by immunofluorescence is a defining feature in the diagnosis of C3 glomerulopathy, a disease entity defined by dysregulation of the alternative complement pathway. Snijders *et al.* [5] recommend the use of C3d in addition to C3c (which is usually more commonly used) in cases suspicious of C3 glomerulopathy.

Boudhabhay *et al.* [24] discovered C3d deposits in the tubules of kidneys of patients with rhabdomyolysis-induced acute kidney injury. C3d staining was surprisingly negative in most of the tested acute tubular necrosis without rhabdomyolysis biopsies, suggesting that complement activation is not a consequence of all tubular injuries, but especially is associated with muscle injuries.

From the point of view of a recent COVID-19

pandemic, it is well known that renal tubular injury may be one of the complications/signs of patients with COVID-19. Pfister *et al.* [25] investigated complement deposition in the tubular compartment and detected complement deposition along the tubular basement membranes. Based on their findings, C3d staining is the strongest in the kidneys of patients who died due to severe COVID-19 infection, indicating that complement-mediated tubular damage might be dependent on the severity of the COVID-19 disease.

C3d immunohistological staining and skin autoimmune diseases

In a study by Magro *et al.* [26], when correlated with the light microscopic and clinical findings, the C3d and C4d assay has significant application in the assessment of select inflammatory skin diseases including vasculopathy conditions, collagen vascular disease, and autoimmune vesiculobullous disorder. From skin diseases, bullous pemphigoid**,** the most common autoimmune blistering disease, may be diagnostically challenging. Bullous pemphigoid usually presents as multiple tense bullae of varying sizes, pruritic urticarial plaques, vesicles, and crusted erosions. These tense bullae of the skin and less oral involvement are due to autoantibodies targeting hemi-desmosomal proteins anchoring the epidermis [27]. To diagnose bullous pemphigoid in patients with compatible clinical manifestations, a skin biopsy is performed from the edge of a recent bulla or the perilesional area. Direct immunofluorescence, indirect immunofluorescence, enzyme-linked immunosorbent assay, and recently, C3d immunohistochemistry, are used as adjuncts to diagnosis [28]. The gold standard for diagnosing bullous pemphigoid is the detection of linear deposition of IgG and/or C3 at the dermo-epidermal junction using direct immunofluorescence. Unfortunately, direct immunofluo-rescence has several disadvantages, primarily the requirement for frozen specimens. Immunohistochemical staining for C3d of paraffin-embedded formalin-fixed tissue samples may be a useful strategy for bullous pemphigoid diagnosis, especially when direct immunofluorescence or ELISA cannot be performed [28,29]. Similarly, according to Pfaltz *et al.* [30], C3d immunohistochemistry is a valuable tool in the diagnosis of bullous pemphigoid of the skin with a sensitivity of at least 97 %. Additionally, C3d immunohistochemistry, when combined with serological studies (enzyme-linked immunosorbent assay), may have practical and cost advantages for bullous pemphigoid diagnosis [31]. We can agree with the conclusion of Al-Shenawy [32] that in the near future, C3d immunohistochemistry could replace direct immunofluorescence in some autoimmune bullous diseases.

The skin biopsy plays an important and powerful role in helping diagnose autoimmune diseases that present with cutaneous findings, such as lupus erythematous, vasculitis, and dermatomyositis. The skin is particularly amenable to biopsy because it is highly accessible. A skin biopsy can be performed bedside with local anesthesia and has minimal risks compared to obtaining a biopsy of internal organs [33]. Lupus erythematosus is a complicated chronic inflammatory disease comprising a spectrum of autoimmune diseases that may affect various organs (systemic lupus erythematosus) or the skin only, without systemic involvement (cutaneous lupus erythematosus). In cutaneous lupus erythematosus, a self-amplifying inflammatory loop is established between cells of both the innate and adaptive immune system. As non-inflammatory cells, keratinocytes contribute to lesional inflammation in cutaneous lupus erythematosus [34]. Recently, C3d immunohistochemistry of skin biopsies may be an important diagnostic adjunct in the evaluation of lupus erythematosus [35]. Immunohistochemical detection of C3d, together with and C4d along the basement membrane zone and CD123 in skin lesions has important diagnostic value for lupus erythematosus [36].

Practical example of Anti-C3d antibodies preparation

Anti-C3d antibodies are a specific type of antibodies that target and bind to the C3d fragment of complement component 3 (C3). C3d is an important protein involved in the complement system, which plays a critical role in immune responses. The complement system helps to recognize and eliminate foreign pathogens, enhance inflammation, and clear immune complexes from the body.

The preparation of anti-C3d antibodies involves several practical steps that are commonly used in antibody production and purification. Here is a practical example of how anti-C3d antibodies can be prepared:

1. Antigen selection: The first step is to identify and isolate the C3d fragment of complement component 3. This can be done by expressing and

purifying recombinant C3d protein or by isolating the natural C3d fragment from a biological source;

2. Immunization: Animals, typically rabbits or mice, are immunized with the purified C3d antigen. The antigen is often mixed with an adjuvant to enhance the immune response;

3. Antibody production: After a suitable immunization period, blood samples are collected from the immunized animals. The blood contains serum, which contains a mixture of various antibodies, including anti-C3d antibodies. The serum is separated from the blood cells through centrifugation;

4. Antibody purification: To obtain pure anti-C3d antibodies, the serum is subjected to antibody purification techniques such as affinity chromatography. Affinity chromatography utilizes a matrix or resin with immobilized C3d antigen, allowing the specific binding and isolation of anti-C3d antibodies from the mixture;

5. Antibody characterization: The purified anti-C3d antibodies are then characterized to confirm their specificity and activity. Techniques such as enzyme-linked immunosorbent assay (ELISA) or Western blotting can be employed to assess the binding affinity and selectivity of the antibodies for the C3d fragment;

6. Antibody storage: The purified anti-C3d antibodies are typically stored in appropriate buffers at low temperatures, such as -20 °C or -80 °C, to maintain their stability and functionality.

Once prepared, anti-C3d antibodies can be used in various research and diagnostic applications (Figs 1-3). They can be employed to study the involvement of the complement system in immune responses, investigate autoimmune disorders, or even develop therapeutic strategies targeting the complement pathway.

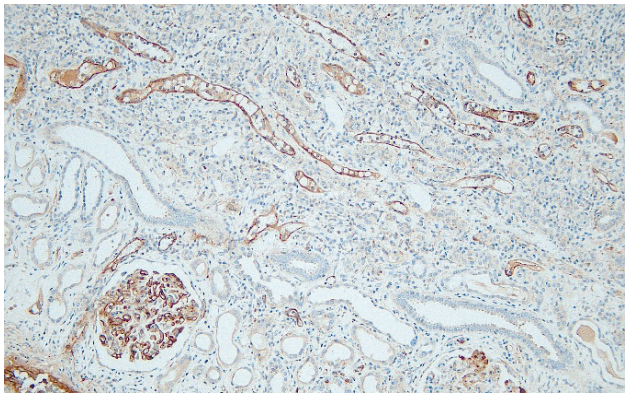

Fig. 1. C3d staining in rejected kidney tissue.

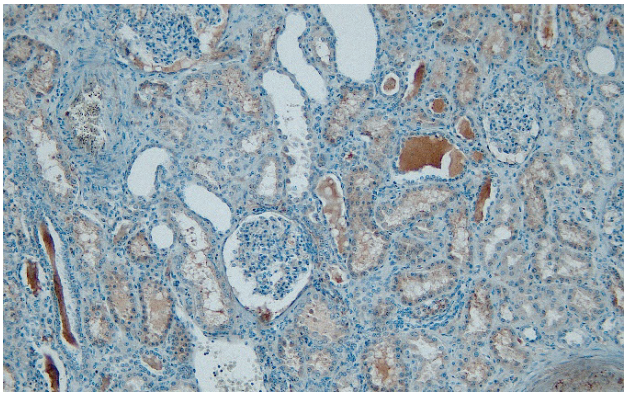

Fig. 2. C3d staining in normal kidney.

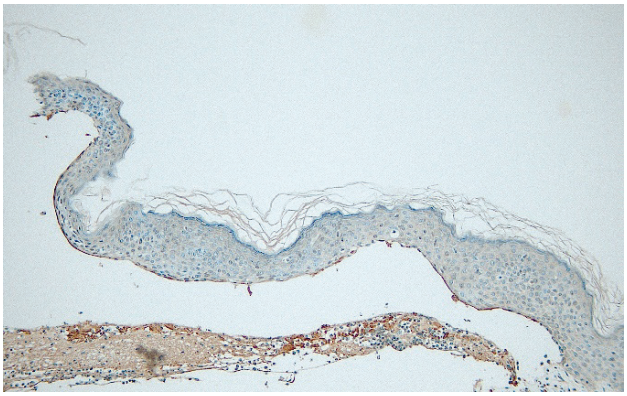

Fig. 3. C3d staining in bullous pemphigoid.

Conclusions and further perspectives

Local C3d is one of the predominant hallmarks in the majority of antibody-mediated rejection patients, indicating upstream complement cascade activation of the classical pathway, which is not reflected by systemic complement activation [37]. The detection of glomerular C3 deposition may be a stable part of the precise diagnosis of renal biopsy tissues, which are routinely stained for C3 fragments. It may be helpful also in histopathological diagnosis of some autoimmune skin diseases, such as the bullous pemphigoid. The antibodies and methods described in this article may advance our ability to detect and monitor tissue C3d deposition. Because C3 fragments are abundant and durable markers of inflammation, they represent a powerful biomarker of tissue inflammation. In the future, quantitative methods of detecting tissue C3 fragment deposits would improve our ability to monitor a patient's disease activity and response to therapy and would advance the application of "personalized medicine" to autoimmune diseases [38].

We have successfully generated monoclonal antibodies against C3d, a degradation product of the third

component of complement C3. Antibodies specific to tissue-bound C3 activation fragments may be employed for targeted delivery of therapeutic and diagnostic agents to sites of tissue inflammation after kidney transplantation or autoimmune skin pathologies. Components of the complement system represent still a neglected part in the possible immunotherapy of autoimmune diseases, but also in the treatment of some hematological malignancies, or some solid cancers [39]. Further investigation of C3d levels in different healthy and diseased human tissue should be considered for potential use in routine patient care, clinical research, and testing of potential novel therapeutic agents.

Conflict of Interest

There is no conflict of interest.

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